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Starch Biosynthesis in Developing Wheat Grain[1]

EVIDENCE AGAINST THE DIRECT INVOLVEMENT OF TRIOSE PHOSPHATES IN THE METABOLIC PATHWAY

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ABSTRACT

We have used ^{13}C-labeled sugars and nuclear magnetic resonance (NMR) spectrometry to study the metabolic pathway of starch biosynthesis in developing wheat grain (*Triticum aestivum* cv Mardler). Our aim was to examine the extent of redistribution of ^{13}C between carbons atoms 1 and 6 of [1-^{13}C] or [6-^{13}C]glucose (or fructose) incorporated into starch, and hence provide evidence for or against the involvement of triose phosphates in the metabolic pathway. Starch synthesis in the endosperm tissue was studied in two experimental systems. First, the ^{13}C sugars were supplied to isolated endosperm tissue incubated *in vitro*, and second the ^{13}C sugars were supplied *in vivo* to the intact plant. The ^{13}C starch produced by the endosperm tissue of the grain was isolated and enzymically degraded to glucose using amyloglucosidase, and the distribution of ^{13}C in all glucosyl carbons was quantified by ^{13}C-NMR spectrometry. In all of the experiments, irrespective of the incubation time or incubation conditions, there was a similar pattern of partial (between 15 and 20%) redistribution of label between carbons 1 and 6 of glucose recovered from starch. There was no detectable increase over background ^{13}C incidence in carbons 2 to 5. Within each experiment, the same pattern of partial redistribution of label was found in the glucosyl and fructosyl moieties of sucrose extracted from the tissue. Since it is unlikely that sucrose is present in the amyloplast, we suggest that the observed redistribution of label occurred in the cytosolic compartment of the endosperm cells and that both sucrose and starch are synthesized from a common pool of intermediates, such as hexose phosphate. We suggest that redistribution of label occurs via a cytosolic pathway cycle involving conversion of hexose phosphate to triose phosphate, interconversion of triose phosphate by triose phosphate isomerase, and resynthesis of hexose phosphate in the cytosol. A further round of triose phosphate interconversion in the amyloplast could not be detected. These data seriously weaken the argument for the selective uptake of triose phosphates by the amyloplast as part of the pathway of starch biosynthesis from sucrose in plant storage tissues. Instead, we suggest that a hexose phosphate such as glucose 1-phosphate, glucose 6-phosphate, or fructose 6-phosphate is the most likely candidate for entry into the amyloplast. A pathway of starch biosynthesis is presented, which is consistent with our data and with the current information on the intracellular distribution of enzymes in plant storage tissues.

Many of the biosynthetic pathways of plant cells are associated with plastids. Thus, starch synthesis in plants is confined to chloroplasts in green photosynthetic tissues and amyloplasts in nongreen storage tissues such as the endosperm tissue of the developing wheat grain. The amyloplast consists of a starch granule and plastid stroma enclosed by a double membrane (3). Based on *in vitro* assays of enzymes presumed to be involved in starch synthesis, a biosynthetic pathway has been proposed for wheat grain (40) and other storage tissues (8, 41, 42). However, although this pathway has received general acceptance, it does not take into account the intracellular compartmentation of starch synthesis within the amyloplast and the selectively permeable nature (44) of plastid membranes. Thus, we cannot confidently say how translocated sucrose is converted into starch.

The transport properties of plastids have been most extensively studied with chloroplasts (12, 13), although there are some reports on the transport properties of chromoplasts isolated from daffodil flowers (23) and of oily plastids isolated from developing endosperm of castor oil seeds (28). All these plastids appear to share the ability to transport 3-phosphoglycerate, DHAP,[4] and, to a lesser extent, glucose. Since amyloplasts and chloroplasts both develop from proplastids, and, under certain conditions, amyloplasts develop into chloroplasts (29) and *vice versa* (2), it has been suggested that triose phosphates are transported into the amyloplast in a manner analogous to that found in chloroplasts (4, 17, 38). Some evidence in support of this concept has been provided by three groups of workers. Using a nonaqueous procedure for amyloplast isolation, Liu and Shannon (24) showed that a soluble extract of a starch granule preparation from maize endosperm contained hexoses, Pi, and glycolytic intermediates, such as hexose phosphates and triose phosphates, consistent with the operation of a glycolytic sequence within this organelle. Using an aqueous isolation procedure, an intact amyloplast fraction has been prepared from soybean suspension cells (25). This fraction contained the plastids' stromal enzymes with little contamination by cytosol or by other organelles. The stromal enzymes included all those needed to convert triose phosphates to starch. More recently, a similar distribution of enzymes was reported for cauliflower bud plastids (19). Further evidence, which is consistent with the concept that triose phosphates are transported into the amyloplast, was provided by MacDonald and ap Rees (26). These authors showed that in suspension cultures of soybean the labeling of starch by [^{14}C]glycerol was appreciable in comparison to that achieved by [^{14}C]sucrose. However, despite the growing

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[4] Abbreviations: DHAP, dihydroxyacetone phosphate; AMG, amyloglucosidase (EC 3.2.1.3); PPi-dependent phosphofructokinase, pyrophosphate: fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90); ATP-dependent phosphofructokinase, 6-phosphofructokinase (EC 2.7.1.11); G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; F1,6bP, fructose 1,6-bisphosphate; PPP, pentose phosphate pathway; PCA, perchloric acid; G3P, glyceraldehyde 3-phosphate.

circumstantial evidence, there have been no reported studies of the transport properties of isolated amyloplasts. Thus, with the presently available evidence, the metabolic pathway of starch synthesis in plant storage tissues remains unknown. The compound that crosses the amyloplast membrane to support starch synthesis could be any intermediate between hexose phosphate and triose phosphate.

In the present study we have used ^{13}C sugars and ^{13}C-NMR spectrometry to study the metabolic pathway of starch synthesis in developing wheat grain. Our aim was to examine the extent of redistribution, resulting from triose phosphate isomerization, of ^{13}C between carbons 1 and 6 of glucose incorporated into starch and hence provide more direct evidence concerning the involvement of triose phosphate in this metabolic pathway.

MATERIALS AND METHODS

Plant Material. A continuous supply of developing wheat (*Triticum aestivum* cv Mardler) grains was achieved by growing approximately 40 seeds each week in John Innes No. 3 compost. After 1 week in a greenhouse with supplementary lighting, using 400 W high pressure sodium lamps to provide long-day conditions, the seedlings were moved to a cold room for vernalization for 6 weeks at 4°C with fluorescent lighting for 8 h/d. The vernalized seedlings were transplanted, two per pot, into 15 cm pots and returned to the greenhouse. Fungal diseases were controlled with Milgo fungicide (ICI, Fernhurst, Surrey, UK), and insect pests using Fumite DDT/Lindane insecticide generators (Pains-Wessex Limited, Salisbury, Wiltshire, UK). After the emergence of the third leaf, the plants were fed (approximately 0.1 g/pot) once every 2 weeks with Phostrogen fertilizer (Phostrogen Limited, Corwen, Clwyd, UK). The developing ears were tagged with the date of anthesis.

Chemicals. D[1-^{14}C]glucose (50–60 mCi·mmol^{-1}, 1.85–2.2 GBq·mmol^{-1}) and D[6-^{14}C]glucose (50–60 mCi·mmol^{-1}, 1.85–2.2 GBq·mmol^{-1}) were purchased from Amersham International PLC (Bucks, UK). D[1-^{13}C]glucose (90*g* atom %) and D[6-^{13}C]glucose (90*g* atom %) were purchased from MSD Isotopes Limited (Cambrian Chemicals, Croydon, UK) or from Amersham International PLC (Bucks, UK). [1-^{13}C]Fructose was synthesized from [1-^{13}C]glucose using immobilized glucose isomerase (obtained from ICI Agricultural Division) and purified by HPLC. All the ^{13}C sugars were checked for isotopic purity by ^{13}C-NMR spectrometry and were more than 90% enriched in the designated carbon. All other chemicals were obtained from either BDH Chemicals Limited (Poole, Dorset, UK) or Sigma Chemical Co. Limited (Poole, Dorset, UK).

***In Vitro* Incubation Conditions.** At various stages of development, wheat ears were removed from the plant by cutting the stem at soil level. The stems were then recut under a 200 mM sucrose solution and the ears illuminated with a 300 W tungsten-halide lamp providing PAR of 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ (measured at ear height using a Crump quantum photometer, Crump Instruments Limited, Billericay, Essex, UK). This technique was adopted in order to prevent a fall in grain sugar levels which occurs when the stems are placed in water in normal laboratory lighting (our unpublished observation). Pairs of grains were removed from the basal florets of the spikelets in the central region of the ear, then weighed and dissected. Two methods of dissection were used: (a) 'half-grains,' grains were cut longitudinally using a surgical scalpel blade, exposing a cut endosperm surface to the incubation medium; (b) 'endosperm-halves,' grains were cut longitudinally as above and the pericarp tissue removed, exposing the whole endosperm surface to the incubation medium. The dissected grains (four grain-halves) were quickly transferred to 25-ml conical respirometer flasks with 3 ml incubation medium containing 10 mM MES (2[*N*-morpholino]ethanesulphonic acid) pH 6.0, 5 mM $MgCl_2$, 60 mM KCl, 0.5% bovine serum albumin, and the labeled sugar, as described in the "Results" section, plus sorbitol to achieve a total sugar: plus sorbitol concentration of 330 mM. The flasks were incubated with gentle shaking for up to 5 h at 25°C. When half-grains were incubated in these conditions, the grains were illuminated with a 300 W tungsten-halide lamp that provided PAR of 300 $\mu E \cdot M^{-2} \cdot s^{-1}$ as described previously (10). When endosperm-halves were incubated in these conditions, there was no requirement for light. At the end of the incubation period the tissue was removed from the incubation medium and rinsed twice with 15 ml water. The pericarp was quickly removed from the half-grains, and the endosperm tissue homogenized and treated as described in "Tissue-Handling Procedures."

***In Vivo* Experimental Conditions.** [^{14}C]- or [^{13}C]-glucose was supplied *in vivo* to developing wheat grains, via a nick in the stem, as described previously (10). Using this technique, labeled sugars are translocated to the grains and are incorporated into starch in the endosperm. Grains were taken from the basal florets of the spikelets in the central region of ears at 1, 3, and 7 d after supplying the label. The endosperm tissue was quickly excized and homogenized and treated as described in "Tissue Handling Procedures."

Tissue Handling Procedures. The isolated endosperm tissue was homogenized using a Polytron vortex homogenizer, in 3 ml of ice-cold 1 M PCA. The homogenates were centrifuged at 4000*g* for 15 min at 4°C, and the supernatant (PCA-soluble fraction) was decanted from the starchy pellet (PCA-insoluble fraction). The pellet was washed three times by resuspending in 3 ml ice-cold distilled water and centrifuging as above. The supernatant of the first wash was combined with the PCA-soluble fraction and subsequent washes discarded. The PCA-soluble fraction was adjusted to pH 6.5 to 7.5 using 5 M KOH and 1 M KH_2PO_4. The insoluble potassium perchlorate was removed by centrifugation at 4000*g* for 10 minutes at 4°C. The PCA-soluble fractions were kept at 4°C during processing.

Analysis of ^{14}C Incorporation into Endosperm Tissue. The washed PCA-insoluble fraction was resuspended in 5 ml 0.1 M acetate buffer (pH 4.5) and boiled for 30 min. After cooling, the gelatinized starch was degraded enzymically to glucose by incubation overnight at 37°C with 14 units AMG (Sigma grade V). Any insoluble material remaining was removed by centrifugation at 4000*g* for 15 min. In some experiments, the washed PCA-insoluble fraction was resuspended in 1 ml 0.1 M acetate buffer (pH 4.5) and incubated for various times at 37°C with 10 units AMG (Miles Scientific, Slough, UK). The Miles AMG enzyme was selected because it was found to be almost free from contamination with β1,3-glucanase activity (when assessed using laminarin, the contamination of the Miles and Sigma enzymes was about 0.001% and 0.1%, respectively). Using this technique the starch granules in the PCA-insoluble fraction are partially hydrolyzed to glucose (this was verified by HPLC analysis, which also showed that the ^{14}C was associated only with glucose). Glucose in the supernatant fraction was measured by the glucose oxidase method (45). A fraction of the supernatant of the enzyme-digested PCA-insoluble fraction or the PCA-soluble fraction was mixed with 10 ml Beckman MPC scintillant and radioactivity measured on a Beckman 8086 liquid scintillation counter. External standards were used to correct for quenching by the H# principle.

Analysis of ^{13}C Incorporation into Endosperm Tissue. The washed PCA-insoluble fraction was resuspended in 1 ml 0.1 M acetate buffer (pH 4.5) and incubated for 45 min at 37°C with 10 units AMG (Miles Scientific, Slough, UK). Residual starch was removed by centrifugation at 10,000*g* for 10 min. Using this technique, it is possible to preferentially hydrolyze a very enriched fraction of labeled starch, which is high enough in ^{13}C content to be detectable by ^{13}C-NMR spectrometry. Sucrose was isolated

by HPLC from the combined PCA-soluble fractions of at least 100 endosperms. The samples were deionized and freeze-dried as described previously (21), and sucrose isolated by HPLC on a Waters Sugar Analyzer 1 (Waters Associates Limited, Northwich, Cheshire, UK) fitted with a Bio-Rad HPX-87C carbohydrate analysis column (300 × 7.8 mm column; column temperature 85°C) and a Bio-Rad Micro-Guard carbohydrate refill cartridge (Bio-Rad Laboratories Limited, Watford, Hertfordshire, UK), with water as the mobile phase at a flow-rate of 0.7 ml/min. Sugars were detected using a Waters R401 Refractive Index monitor coupled to a Trivector TriLab 2000 chromatography data system (Trivector Scientific Limited, Sandy, Bedfordsire, UK). Prior to NMR analysis, the glucose released from starch samples and HPLC-purified sucrose samples were filtered using a 0.45 μm Acrodisc filter (Gelman Sciences Inc., Brackmills, Northampton, UK).

NMR Spectrometry of ^{13}C-Glucose from Starch or ^{13}C-Sucrose. ^{13}C-NMR spectra were obtained on a JEOL FX 100 NMR spectrometer (Jeol [UK] Ltd., Collindale, London) operating at a carbon frequency of 25.00 MHz. The samples were in water solution with a small amount of $(CD_3)_2CO$ for field/frequency locking and contained in 10 mm NMR tubes. The carbon 90° pulse was at most 28 μs, so that off-resonance effects over the width of the spectrum were negligible. The spectra were obtained with gated proton decoupling so that the spectra do not include nuclear Overhauser enhancement effects. Although all the carbons considered have directly attached protons, the spectra are nonetheless quantitative. This assertion was proven to be correct on a sample without enrichment. The recycle time was 3 s. Chemical shifts were referred to the acetone methyl resonance at 29.8 ppm.

RESULTS

Partial Hydrolysis of Starch. Preliminary experiments (data not presented) investigated isotope incorporation using ^{14}C. When the starch was completely digested by AMG to glucose, we found that the specific activity of the glucose released by hydrolysis was too low to be detectable by ^{13}C-NMR spectrometry. This problem was circumvented by the development of a technique which partially hydrolyzes the starch to release preferentially the labeled glucose from the starch granules. Using ^{14}C-starch from endosperm that had previously been incubated with [^{14}C]glucose, we found that incubation with AMG without prior gelatinization preferentially released labeled glucose of high specific activity from the starch granules (Fig. 1). Digestion for 45 min released 80% of the labeled glucose but left 90% of the total starch granule undigested. The level of isotope incorporation into this label-enriched digest was now sufficient for quantitative assessment by NMR spectrometry of ^{13}C incorporation into starch. Without this development, our ^{13}C experiments would not have been possible.

Distribution of ^{13}C and ^{14}C in Sucrose and Starch Synthesized at Various Stages of Endosperm Development. Preliminary *in vitro* experiments using [1-^{14}C]glucose showed minimal label incorporation at 8 d post-anthesis (21.6 ± 2.2 nmol glucose equivalents/h), maximal label incorporation at 14 and 19 d post-anthesis (respectively: 80.5 ± 3.4 and 101.0 ± 2.6 nmol glucose equivalents/h), with decreasing ^{14}C incorporation into starch at 25, 29, and 36 d post-anthesis (respectively: 52.0 ± 0.9, 20.0 ± 0.8, and 19.1 ± 0.8 nmol glucose equivalents/h). The rate at the maximal level of label incorporation into starch was approximately 0.4 mg/d; which is equivalent to about half the rate of starch deposition *in vivo*. When [1-^{13}C]glucose was supplied in place of [1-^{14}C]glucose, incorporation of ^{13}C could not be detected by ^{13}C-NMR spectrometry before 15 d and was barely detectable after 25 d post-anthesis. Thus, despite the technique which partially hydrolyzes the starch, our NMR analysis of

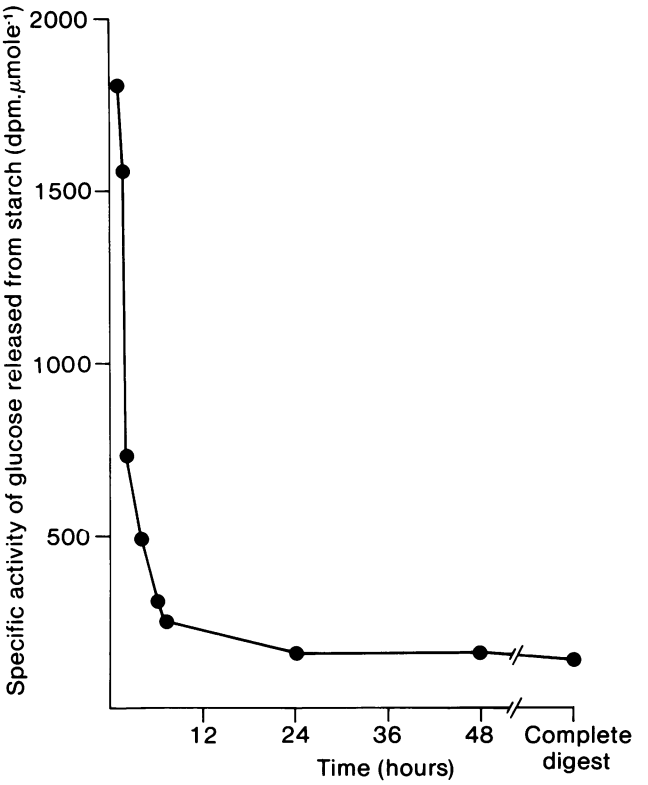


FIG. 1. Specific activity of ^{14}C-labeled glucose released from starch granules incubated with AMG. Starch granules were obtained from endosperm tissue, from 10 grain at 21 d post-anthesis, previously incubated at 25°C with [1-^{14}C]glucose for 5 h. The ^{14}C-starch, produced by metabolism in the endosperm tissue, was extracted after homogenizing the tissue in the ice-cold 1 M perchloric acid. The starch granules were washed with water and partially hydrolyzed to glucose using AMG. Results are from individual determinations taken sequentially from one batch of starch granules.

[^{13}C]glucose incorporation into starch was only possible over the period of most rapid starch synthesis (15–25 d post-anthesis) when label incorporation was greatest. All subsequent experiments reported in this paper were done at 21 d post-anthesis.

Analysis of ^{13}C Distribution in Glucose Released from Starch. Figure 2a shows the ^{13}C-NMR spectrum of 'Analar' glucose. The resonances have been assigned on the basis of chemical shift information for pure glucose (18). Figure 2, b and c, shows the ^{13}C-NMR spectra of glucose released from starch extracted from endosperm tissue which had been incubated with [1-^{13}C]- or [6-^{13}C]glucose. There was an increased incidence of ^{13}C in carbons 1 and 6 and no detectable increase in the incidence of ^{13}C in carbons 2 to 5. Most of the label remained in its original position. Peak areas from the spectra were normalized to the mean values for carbons 2 to 5. Figure 3 shows the within-experiment variation (*i.e.* several identical incubations were done on the same day, with each incubation containing endosperm tissue from different plants) in ^{13}C incidence in carbons 1 to 6 of glucose released from starch extracted from endosperms incubated with 10 mM [1-^{13}C]glucose. For this experiment, there was very little within-experiment variation in the ratio of ^{13}C incorporated into carbon 6 *versus* carbon 1 (14.98% ± 0.12). Over a period of months, however, it was noticed that there was some between-experiment variation (*i.e.* identical incubations done on different days) in the percentage redistributed. Because of this variability, it was thought necessary to design experiments with different incubation conditions set up on the same day with endosperm tissue taken from a single batch of plants. With this

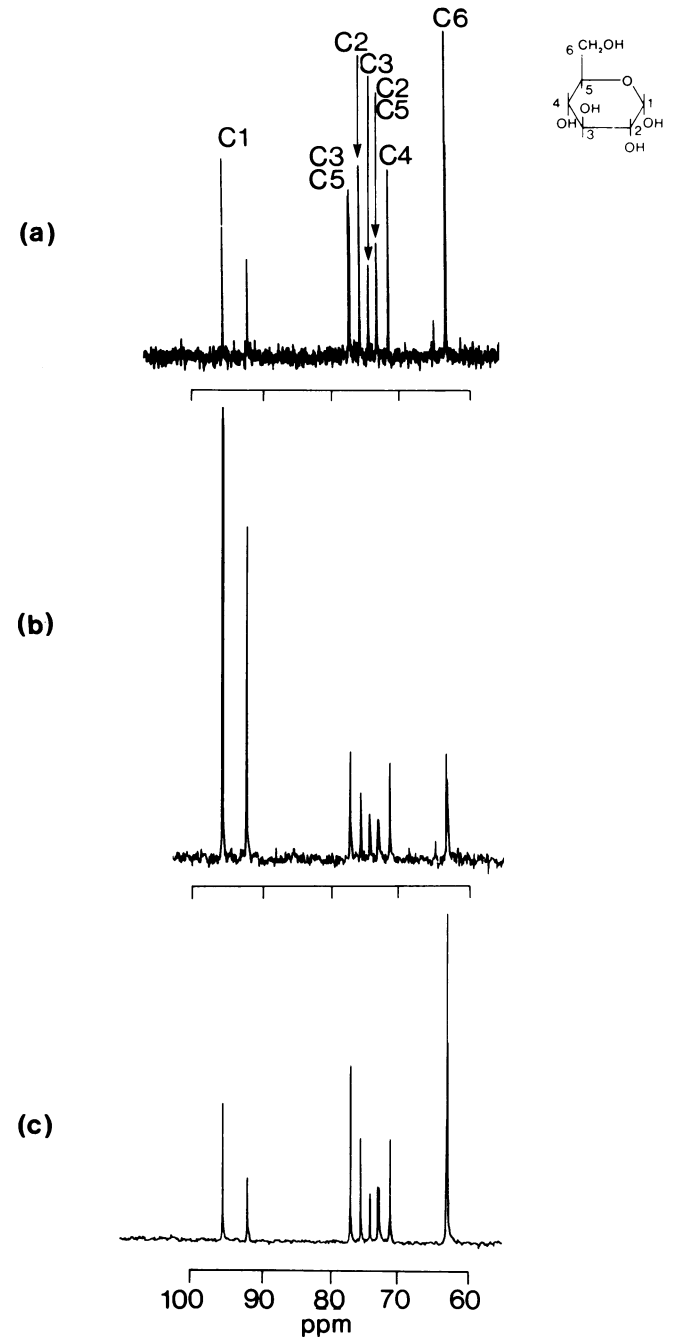


FIG. 2. a, ^{13}C-NMR spectrum of 'Analar' glucose showing carbon assignments. b, ^{13}C-NMR spectrum of glucose released from starch. Endosperm tissue from 10 grain at 21 d postanthesis was incubated for 5 h at 25°C with [1-^{13}C]glucose. The ^{13}C-starch, produced by metabolism in the endosperm tissue, was extracted after homogenizing the tissue in ice-cold 1 M perchloric acid. The starch was partially hydrolyzed to glucose using AMG. c, ^{13}C-NMR spectrum of glucose released from starch isolated from endosperm tissue incubated with [6-^{13}C]glucose.

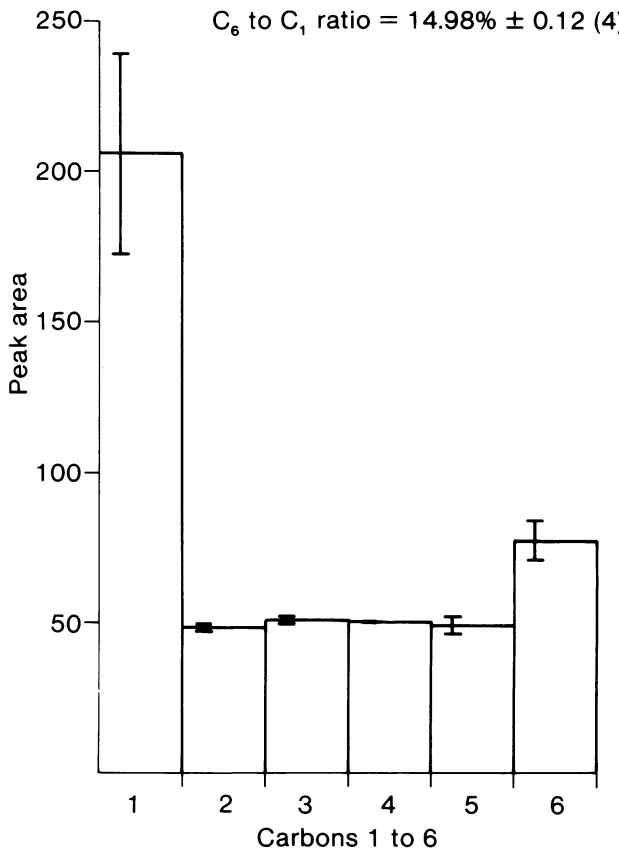


FIG. 3. ^{13}C-carbon distribution in all carbon atoms of glucose released from starch. Isolated endosperm tissue from 10 grain at 21 d post-anthesis was incubated for 5 h at 25°C with [1-^{13}C]glucose. The ^{13}C-starch (produced by metabolism in the endosperm tissue) was isolated, after homogenizing the tissue in the ice-cold 1 M perchloric acid. The starch was partially hydrolyzed to glucose using AMG. The distribution of ^{13}C in glucose hydrolyzed from starch was measured by NMR spectrometry. Results are expressed as the mean ± SE of four determinations.

experimental design, significant differences were detectable between different incubation conditions (see below and Table I).

When treatments (1 h *versus* 5 h incubation periods, aerated *versus* anoxic incubations, 10 mM glucose incubated with and without 100 mM sucrose, 20 mM glucose incubated with and without 20 mM fructose) were applied that altered the amount of label incorporated into starch, the patterns of redistribution of ^{13}C between C1 and C6 of glucose released from starch remained unaltered (Table I). This was also the case when different concentrations of glucose were supplied at 25 and 15°C: these treatments altered the amount of label incorporated into starch (results not shown) but did not affect the redistribution of ^{13}C from C1 to C6 of [1-^{13}C]glucose incorporated into starch (for incubations with 10, 20, 50, 70, and 100 mM glucose, the respective redistribution ratios were 15.9, 21.0, 13.7, 15.0, and 20.6% at 15°C and 14.6, 15.7, 12.7, 18.1, and 17.8% at 25°C). A similar degree of redistribution of label was also observed when [1-^{13}C]fructose was supplied in place of [1-^{13}C]glucose (Table I).

Analysis of ^{13}C and ^{14}C Distribution in Sucrose and Starch Extracted from Endosperm Incubated *in Vitro*. Resonances have been assigned to the ^{13}C-NMR spectra of sucrose (Fig. 4a) on the basis of chemical shift information for sucrose (18). Figure 4b shows the ^{13}C-NMR spectra of sucrose extracted and purified from endosperm tissue which had been incubated with [1-^{13}C]glucose with an equimolar concentration of unlabeled fructose. Peak areas for these spectra were normalized to the mean values for carbons 2 to 5 of both the glucosyl and fructosyl moieties. There was an increased incidence of ^{13}C in carbons 1 and 6 of both the glucosyl and fructosyl moiety and no evidence of any increased incidence of ^{13}C in carbons 2 to 5. Most of the label remained in its original position.

When an equimolar concentration of unlabeled fructose was added to incubations with [1-^{14}C]glucose, less label was incor-

Table I. *Effect of Different Incubation Conditions on the Percentage C1:C6 Label Redistribution Ratios in Glucose Recovered from Starch*

Isolated endosperm tissue or grain bisected longitudinally through the crease region was incubated *in vitro* for 5 h at 25°C with [1-^{13}C]glucose. The different incubation conditions significantly altered the overall flux of the label to starch. The ^{13}C-starch (produced by starch synthesis in the endosperm tissue) was harvested, after homogenizing the tissue in the 1 M perchloric acid and partially hydrolyzing the starch to glucose using AMG. The distribution of ^{13}C in all glucosyl carbons was measured by NMR spectrometry. Results are expressed as the mean of one determination, with paired samples prepared from 10 randomly selected grains of ear at 21 d postanthesis.

Incubation Conditions	Starch Synthesis Rate/ Endosperm	Percent ^{13}C Redistribution in Starch from C1 to C6
	nmol glucose equivalents incorporated into starch/5 h[a]	%
Endosperm from half-grains *in vitro*		
100 mM glucose [1-^{13}C]		
5 h incubation	1032.0	14.1
1 h incubation	175.6	13.9
100 mM glucose [1-^{13}C]		
Aerated incubation	1032.0	22.7
Anoxic incubation	76.5	23.4
10 mM glucose [1-^{13}C]		
Incubated without 100 mM sucrose	—[b]	22.7
Incubated with 100 mM sucrose	—[b]	20.8
Endosperm halves incubated *in vitro*		
20 mM glucose [1-^{13}C]		
Incubated without 20 mM fructose	844.0	15.0
Incubated with 20 mM fructose	211.5	15.0
20 mM glucose [1-^{13}C]	844.0	14.4
20 mM fructose [1-^{13}C]	166.5	12.4

[a] Endosperm supplied with [1-^{14}C]glucose, starch degraded completely with AMG. [b] Not determined.

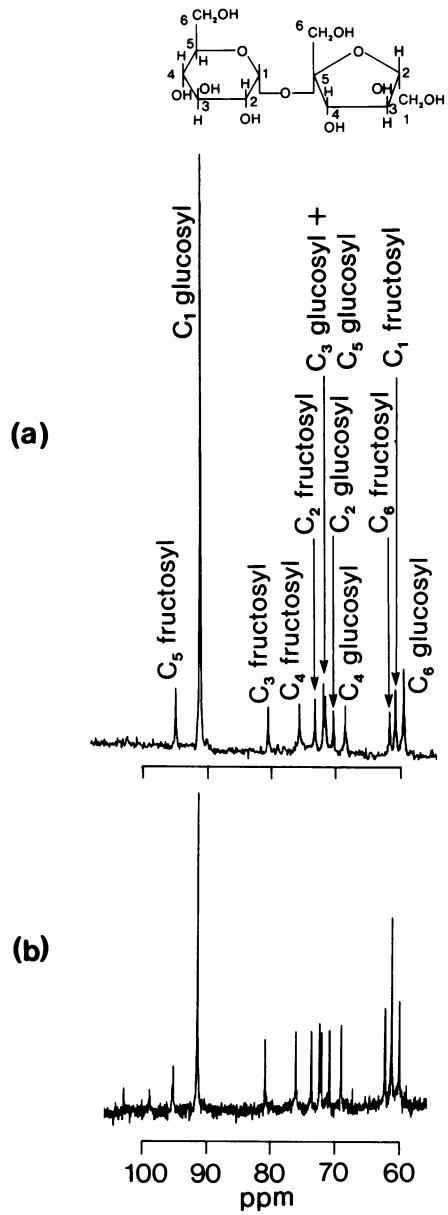


FIG. 4. a, ^{13}C-NMR spectrum of 'Aristar' sucrose showing carbon assignments; b, ^{13}C-NMR spectrum of sucrose isolated from endosperm tissue. Endosperm tissue from 400 grains at 21 d post-anthesis was incubated for 5 h at 25°C with [1-^{13}C]glucose. The [^{13}C]sucrose, produced by metabolism in the endosperm tissue, was extracted after homogenizing the tissue in ice-cold 1 M perchloric acid and was purified by HPLC.

porated into starch and there was a greater proportion of label in the fructosyl moiety of sucrose (data not presented). When the mixture of hexoses was supplied, 43% of the ^{13}C in sucrose (Table II) was present in the fructosyl moiety (35% when [^{14}C]glucose was used, data not presented), compared with 8% of the ^{13}C when glucose alone was supplied (11% when [^{14}C]glucose was used, data not presented). Thus, the partitioning of label was altered by the addition of fructose to the incubation medium and yet the percentage redistribution of ^{13}C was very similar in each treatment in the glucose recovered from starch and in both hexosyl moieties of sucrose (Table II). These two experiments were not done at the same time, with the same batch of plants, which may explain why there was a difference between these two experiments in the C1 to C6 redistribution ratios in starch (19.3% compared with 8.8%). In our experience, the exact degree of redistribution varies and is a characteristic of the batch of plants used on that day. Thus, there was no difference in the ratio when this experimental comparison was done on the same day with the same batch of plants (Table I).

When [6-^{13}C]glucose was supplied, the percentage redistribution between carbons 1 and 6 of glucose incorporated into starch (8.1%) was significantly lower ($P < 0.01$) than that observed with [1-^{13}C]glucose supplied to the same batch of plants in the same experiment (17.7%) (Table III). The percentage redistribution within the hexosyl moieties of sucrose was similar when [1- or 6-^{13}C]glucose was supplied (17.9% compared with 15.0%, respectively, Table III). The distribution of label between the glucosyl and the fructosyl moieties of sucrose was identical when [1- or 6-^{13}C]glucose was supplied.

Analysis of ^{13}C and ^{14}C Carbon Distribution in Sucrose and Starch Extracted from Endosperm of Grain Supplied *in Vivo* with [1-^{13}C]- or [1-^{14}C]Glucose. Over a 7-d period after supplying grains *in vivo* with [1-^{14}C]glucose, the proportion of label in the soluble fraction of the endosperm tissue declined from 32% at d 1 to 8% at d 7 (data not presented). Furthermore, the distribution of label present in the neutral fraction of the soluble extract altered. Thus, at d 1, 55% of the label was present as sucrose, whereas by d 7 this proportion had declined to 27%. The pro-

Table II. *In Vitro Experiment with 20 mM [1-^{13}C]Glucose With and Without 20 mM Fructose:Percentage Redistribution Ratios in Sucrose and Starch Extracted from Endosperm*

Isolated endosperm tissue from 400 grain at 21 d post-anthesis was incubated *in vitro* for 5 h at 25°C with [1-^{13}C]glucose or with [1-^{13}C]glucose plus unlabeled fructose. The ^{13}C-starch and [^{13}C]sucrose (produced by metabolism in the endosperm tissue) was isolated, after homogenizing the tissue in the ice-cold 1 M perchloric acid. The starch was partially hydrolyzed to glucose using AMG, and the sucrose was purified by HPLC. The distribution of ^{13}C in glucose hydrolyzed from starch or purified sucrose was measured by NMR spectrometry. Results are expressed as the mean of one determination from 400 grain.

		Percent ^{13}C Redistribution from C1 to C6	
		[1-^{13}C]glucose only	[1-^{13}C]glucose plus fructose
Sucrose	Glucosyl:Fructosyl	57.43[a]	92:8[a]
		%	
Sucrose	Glucosyl C1:C6	15.9	10.1
Sucrose	Fructosyl C1:C6	17.1	11.8
Sucrose	Average C1:C6	16.5	11.0
Starch	(Glucose) C1:C6	19.3	8.8

[a] Glucosyl to fructosyl ratio.

Table III. *In Vitro Experiment with 20 mM [1-^{13}C]- or [6-^{13}C]Glucose:Percentage Redistribution Ratios in Sucrose and Starch Extracted from Endosperm*

Isolated endosperm tissue from 100 grain at 21 d post-anthesis was incubated *in vitro* for 5 h at 25°C with [1-^{13}C] or [6-^{13}C]glucose. The ^{13}C-starch and [^{13}C]sucrose (produced by metabolism in the endosperm tissue) was isolated, after homogenizing the tissue in the ice-cold 1 M perchloric acid. The starch was partially hydrolyzed to glucose using AMG and the sucrose was purified by HPLC. The distribution of ^{13}C in glucose hydrolyzed from starch or purified sucrose was measured by NMR spectrometry. Results of sucrose measurements are expressed as the mean of one determination from 100 grain.

		Percent ^{13}C Redistribution from C1 to C6	
		[1-^{13}C]glucose only	[6-^{13}C]glucose only
Sucrose	Glucosyl:Fructosyl	58:42[a]	55.45[a]
		%	
Sucrose	Glucosyl C1:C6	16.0	15.2
Sucrose	Fructosyl C1:C6	19.7	14.7
Sucrose	Average C1:C6	17.9	15.0
Starch	(Glucose) C1:C6	17.7 (±1.0)[b]	8.1 (±0.1)

[a] Glucosyl to fructosyl ratio. [b] Data represent the mean ± SE with four replicates per determination.

portion of label in glucose was always much lower than this (13% at d 1 and 7% at d 7) whereas the proportion of label in oligosaccharides (mainly fructans) was 12% at d 1 but higher (34 and 36%) at d 3 and 7 (data not presented).

One day after supplying [1-^{13}C]glucose, both the glucosyl and fructosyl moieties of sucrose were labeled. The distribution of label between C1 and C6 was similar in sucrose and in starch (25.5% compared with 27.8%, Table IV), although the percentage redistribution between C1 and C6 in the glucosyl and fructosyl moieties of sucrose was apparently not identical (21.8 compared with 29.1%). At 3 d after supplying [1-^{13}C]glucose, the redistribution of label between carbon 1 and 6 in starch was similar to that seen at 1 day after supplying the label. The percentage redistribution between carbons 1 and 6 of label incorporated into the glucosyl moiety of sucrose was lower at 3 d than at 1 d after supplying the [1-^{13}C]glucose. We were unable to obtain accurate data for starch or sucrose by 7 d after supplying the label.

Table IV. *In Vivo Experiment with [1-^{13}C]Glucose:Percentage Redistribution Ratios in Sucrose and Starch Extracted from Endosperm*

Plants at 21 d post-anthesis were supplied with [1-^{13}C]glucose via a nick in the stem. One and 3 d after supplying the label, the endosperm tissue was dissected from the grain. The ^{13}C-starch and [^{13}C]sucrose (produced by metabolism in the endosperm tissue) was isolated, after homogenizing the tissue in the ice-cold 1 M perchloric acid. The starch was partially hydrolyzed to glucose using AMG and the sucrose was purified by HPLC. The distribution of ^{13}C in glucose hydrolyzed from starch or purified sucrose was measured by NMR spectrometry. Results are expressed as the mean of one determination from 40 grain.

		Percent ^{13}C Redistribution from C1 to C6	
		d 1	d 3
Sucrose	Glucosyl:Fructosyl	57:43[a]	55:45[a]
		%	
Sucrose	Glucosyl C1:C6	21.8	16.2
Sucrose	Fructosyl C1:C6	29.1	—[b]
Sucrose	Average C1:C6	25.5	16.2
Starch	(Glucose) C1:C6	27.8 (±1.1)[c]	23.8

[a] Glucosyl to fructosyl ratio. [b] Very low ^{13}C levels in carbon 6—data inaccurate. [c] Data represent the mean ± SE of four replicate incubations.

Interestingly, using [^{14}C]glucose we found that by 1 d after supplying the label most of the radioactivity in the neutral fraction of the soluble extract was present as sucrose (55%) and only 13% was present as glucose. This result, together with the finding that the proportion of label in sucrose declined from 55% at d 1 to 27% at d 7 suggests that the [^{13}C]glucose may have been converted to sucrose in the stem before being delivered to the grains. We confirmed that this was the case by supplying [^{14}C]glucose and analyzing the contents of the endosperm cavity: all the ^{14}C recovered from the cavity was associated with sucrose (data not presented). Thus, although in all our experiments labeled glucose was supplied, in the *in vivo* experiments the label had been converted to sucrose before being made available for starch synthesis in the endosperm tissue.

DISCUSSION

Redistribution of Label between the Hexosyl Moieties of Sucrose. When [1-^{14}C]glucose was supplied *in vitro* to developing wheat grains, the label accumulated in starch and in sucrose. There was some redistribution of radioactivity between the hexosyl moieties of sucrose. This has been reported previously when wheat grains were supplied with asymmetrically labeled sucrose (35) and is thought to be due to sucrose hydrolysis and resynthesis from redistributed glucosyl and fructosyl intermediates in the endosperm. The presence and absence of unlabeled fructose in the incubation medium altered the proportion of label accumulated in starch and also affected the distribution of label between the hexosyl moieties in sucrose. These changes in the proportions of label in the hexosyl moieties of sucrose reflect the reversible nature of some of the reactions in this part of the metabolic pathway. Furthermore, there can be movement of label without net synthesis. Thus, label incorporation into sucrose can proceed via sucrose P synthase and sucrose phosphatase and also via the reversible activity of sucrose synthase. The hex-

ose moieties used for sucrose synthesis are made available via the reversible activities of UDP-glucose pyrophosphorylase, phosphoglucomutase, and hexose phosphate isomerase and the irreversibility of the hexokinase reaction. When fructose is absent, redistribution of label is inevitable as label incorporation into sucrose would proceed largely from UDP-glucose and F6P pools which are apparently in equilibrium. When fructose is added, along with labeled glucose, the largely irreversible activity of hexokinase combined with the reversible activity of sucrose synthase allows label incorporation into sucrose to proceed from unlabeled fructose pools and labeled UDP-glucose pools.

Relevance of Redistribution between C1 and C6 of Glucose Incorporated into Starch to the Involvement of Triose Phosphates in the Metabolic Pathway. In this work it has been assumed that the triose phosphates, G3P and DHAP, are in equilibrium within the cell and that any resynthesis of F1,6bP from triose phosphates occurs with random triose units. However, in mammalian tissues (20) there is some evidence that the triose phosphates are not in complete equilibrium. In plant cells there is little conclusive evidence, although what little data are available suggest that they may not be, at least in avocado fruit (22). We have tried to estimate directly the rate of triose phosphate isomerization in wheat endosperm tissue by studying the pattern of redistribution of [2-^{13}C]glycerol incorporated into starch. Unfortunately, this approach was unsuccessful because the level of glycerol incorporation into starch was too low to be detectable by NMR spectrometry. The activity of triose phosphate isomerase in wheat endosperm tissue was found to be 342 μmol·min^{-1}·g fresh weight^{-1} (G Entwistle, personal communication). Since the activity of this enzyme is approximately 2000-fold in excess of the rate of starch synthesis, we suggest that in developing wheat endosperm there is likely to be full isotopic equilibrium between the triose phosphates. Thus, if triose phosphates are involved in the metabolic pathway of starch synthesis, starch synthesized from glucose labeled specifically in the C-1 or C-6 positions should be found to have a high degree of redistribution of label between the C-1 and C-6 carbons. Conversely, if the metabolic pathway of starch biosynthesis involves a series of hexose interconversions, without the direct involvement of triose phosphates, starch synthesized from such asymmetrically labeled glucose should be found to have a highly conserved pattern of labeling.

When [1-^{13}C]fructose or glucose was supplied *in vitro* to endosperm tissue or to 'half-grains,' label accumulated in starch. Analysis by ^{13}C-NMR spectrometry of the label distribution pattern in the glucosyl carbons of starch showed that the ^{13}C remained predominantly in the originally supplied carbon (75–85%), although there was some redistribution between the C-1 and C-6 carbons (15–25%). We were unable to detect any increase in the redistribution of label in the other glucosyl carbons of starch. This partial redistribution of label was remarkably constant despite different incubation conditions which altered the partitioning of ^{14}C between the soluble and insoluble fractions of the endosperm tissue. A similar degree of partial redistribution of label was obtained when [1-^{13}C]glucose was supplied *in vivo* via a nick in the stem. Thus, in all the experiments there was only a partial degree of redistribution between the C1 and C6 carbons of label recovered from starch.

Our results show that triose phosphates are at least in part involved in the pathway of starch biosynthesis. However, although these findings are consistent with the hypothesis that the carbon compounds transported into the amyloplast may be triose phosphates (4, 17, 19, 24–26, 31, 38), an alternative explanation is that the involvement of triose phosphates is a result of a pathway cycle between hexose phosphates and triose phosphates in the cytosol. We examined this possibility by studying the redistribution of label in sucrose. Sucrose is most unlikely to be synthesized in the amyloplast (25) but is synthesized from hexoses in the cytosol, as described above, by the enzymes sucrose synthase or sucrose P synthase and sucrose phosphatase (1). Thus, triose phosphates can be implicated directly in the pathway of starch synthesis only if the degree of redistribution of label between C1 and C6 found in starch significantly exceeds that found in the hexosyl moieties of sucrose.

The C1 to C6 redistribution ratios were the same in the hexosyl moieties of sucrose and in glucose released from starch recovered from endosperm tissue supplied *in vitro* and *in vivo* with [1-^{13}C]glucose. Thus, in terms of their C1 to C6 redistribution ratios, the intermediates which are available for sucrose synthesis (UDP-glucose, F6P, and fructose) are apparently in equilibrium with the precursors available for starch synthesis (ADP-glucose). We believe these data seriously weaken the argument for the direct involvement of triose phosphates in the pathway of starch biosynthesis from sucrose. This proposal that triose phosphates are not involved directly in the pathway of starch synthesis in developing wheat grain is founded on the following logic: first, the enzymes available for sucrose synthesis are almost certainly excluded from the amyloplast, while the enzymes available for starch synthesis are confined to the amyloplast (19, 25); second, triose P isomerase is located in both the amyloplast and in the cytosol (19, 25); and third, if a triose phosphate is transported into the amyloplast, the triose phosphate isomerase present in that compartment should lead to further C1 to C6 redistribution, such that the hexoses available for starch synthesis in the amyloplast would not appear to be so consistently in equilibrium with the hexoses available for sucrose synthesis in the cytosol. For these reasons, it seems improbable that a triose phosphate is transported into the amyloplast as a significant proportion of the main flux of carbon from sucrose to starch. Instead, we suggest that a more likely candidate for transport into the amyloplast is a hexose monophosphate such as G1P, F6P, or G6P.

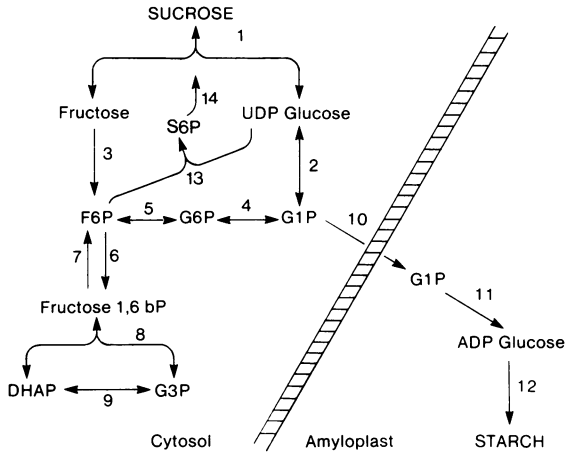
The finding that there was some redistribution between C1 and C6 of label incorporated into sucrose suggests that there is a cytosolic pathway cycle between hexose phosphates and triose phosphates. A relatively constant proportion, amounting to 30 to 40% of the hexose sugars, appears to enter the proposed cytosolic pathway cycle. Due to triose phosphate isomerase activity, label redistribution occurs between the triose phosphates (C1 and C6 of the resynthesized F1,6bP) to give a range of redistribution ratios in the cytosolic hexoses of between 15 and 20%. The proportion of pathway cycling appears to remain constant, despite changes in the flux of glucose to starch. Interestingly, other workers have reported that the proportion of label redistributed between C1 and C6 carbons is remarkably constant in several plant species and plant parts (5, 9, 27, 30, 36, 37, 39, 43). To our knowledge, this is the first suggestion of this pathway cycle in plant cells, but the finding is analogous to that proposed in animal tissues between F6P and F1,6bP (7, 34). We therefore speculate that this pathway cycle is of some importance to the tissue, perhaps by providing PPi, from PPi-dependent phosphofructokinase, at the expense of ATP, from ATP-dependent phosphofructokinase. This possibility would be consistent with the proposal (16, 33) that PPi could be provided in the cytosol for UDP-glucose pyrophosphorylase by PPi-dependent phosphofructokinase. The reported allosteric properties of the phosphofructokinase reactions reviewed by Huber (15) could present a mechanism for controlling the pathway cycle in response to changes in the flux of carbon to starch.

Relevance of the Redistribution of [6-^{13}C]Glucose Incorporated into Starch and Sucrose. The data show a significantly lower percentage label redistribution in glucose recovered from starch synthesized in endosperm supplied with [6-^{13}C]glucose than was the case when [1-^{13}C]glucose was provided. We suggest that the interpretation of our data for ^{13}C incorporation into starch is complicated by the preferential loss of label from C1 of hexose

via the oxidative pentose phosphate pathway, PPP, in the amyloplast. This leads to an underestimate of the proportion of ^{13}C in the C1 carbons of glucose incorporated into starch. Therefore, the redistribution ratio in starch for [1-^{13}C]glucose (82:18) is an overestimate, while the ratio for [6-^{13}C]glucose (92:8) is an underestimate of the 'true' ratio which may lie somewhere between these two estimates. If this difference does arise because of losses of C1 in the oxidative PPP, the activity of this pathway is apparently substantial relative to the rate of starch synthesis. Furthermore, since the C1 to C6 ratio in sucrose was similar when [1- or 6-^{13}C]glucose was supplied, we suggest that most of the PPP activity must be located in the amyloplast. Although there have not been any previous reports of an active PPP for wheat grain, other authors using several plant tissues have attributed the preferential incorporation of [6-^{14}C]glucose into water-insoluble compounds as evidence for PPP activity in plants (for review, see Ref. 32). We have made similar observations with wheat endosperm showing that 1.5-fold more $^{14}CO_2$ was released from [1-^{14}C]glucose than from [6-^{14}C]glucose. Also, the preferential release of $^{14}CO_2$ from [1-^{14}C]glucose was stimulated by methyl viologen (our unpublished observations). These observations demonstrate the existence of an active PPP in endosperm tissue, and our findings described above suggest that a significant proportion of the PPP activity of endosperm tissue is located in the amyloplast. This proposal would be consistent with the report that several enzymes of the PPP are present in cauliflower bud plastids (19). We also concur with the proposal made by Journet and Douce that the enzymic capacities of amyloplasts permit oxidation to CO_2 and triose phosphate via the PPP with complete, partial, or total restriction of recycling of hexose phosphate. The ^{13}C studies reported here indicate that there may be significant flux through this PPP in the amyloplast.

CONCLUSIONS

Based on the data presented in this paper, and on the available published literature on enzymes present in endosperm tissue and their intracellular compartmentation of enzymes in soybean cells (11, 25) and cauliflower buds (19), we suggest that the metabolic pathway of starch synthesis in developing wheat grain is as shown in Figure 5. Fructose and UDP-glucose are the first products of sucrose breakdown by sucrose synthase. Sucrose hydrolysis by invertase cannot be excluded but seems unlikely to be involved as a major source of cytosolic hexose because invertase activity is very much lower than sucrose synthase and has been reported to be localized almost exclusively in the pericarp tissues of the grain (6). This possibility may also be consistent with the finding that wheat germ hexokinase preferentially phosphorylates fructose rather than glucose (14). G1P formation from UDP-glucose can be catalyzed by the cytosolic enzyme (25) UDP-glucose pyrophosphorylase, a reaction which requires PPi. Two further enzymes, phosphoglucomutase and hexose phosphate isomerase are then responsible for the interconversion of G1P, G6P, and F6P. Resynthesis of sucrose from these cytosolic hexose phosphates then occurs via sucrose synthase or sucrose phosphate synthase and sucrose phosphatase. From our findings, a seemingly constant proportion of 30 to 40% of the hexose present in the endosperm enters a cytosolic pathway cycle between the hexose phosphates and triose phosphates where some C1 to C6 redistribution occurs. One of the now partially redistributed hexose phosphates is then transported into the amyloplast, where it is incorporated into starch via the amyloplastic enzymes (25) ADP-glucose pyrophosphorylase and starch synthase. The recent observation that enzymes of the PPP and glycolysis are partly located in the amyloplast (19), together with the evidence reported above for an active PPP in the amyloplast, may provide an explanation for the presence in the amyloplast of those enzymes capable of converting G1P to triose phosphates (19, 25).



Enzymes

1 Sucrose synthase
2 UDP - glucose pyrophosphorylase
3 Hexokinase
4 Phosphoglucomutase
5 Hexose phosphate isomerase
6 ATP-dependent phosphofructokinase
7 Fructose - 1, 6-bisphosphatase and PPi-dependent phosphofructokinase
8 Aldolase
9 Triose phosphate isomerase
10 Hexose translocator
11 ADP - glucose pyrophosphorylase
12 Starch synthase
13 Sucrose phosphate synthase
14 Sucrose phosphatase

FIG. 5. Proposed metabolic pathway of starch synthesis.

The uncertainty as to which hexose compound is transported into the amyloplast may only be resolved with studies of the transport properties of isolated functional amyloplasts.

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